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Tombusvirus replication depends on Sec39p endoplasmic reticulum-associated transport protein

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ABSTRACT

Positive-stranded RNA viruses subvert subcellular membranes to built viral replicases complexes (VRCs) in infected cells. Tombusviruses use peroxisomal membranes for the assembly of their VRCs and they can efficiently switch to the endoplasmic reticulum membrane in the absence of peroxisomes. In this paper, we show that the ER-resident Sec39p vesicular transport protein is critical for the formation of active VRCs in yeast model host. Repression of Sec39p expression in yeast or in plants resulted in greatly reduced tombusvirus accumulation. Moreover, the purified tombusvirus replicase from Sec39p-depleted yeast cells showed low *in vitro* activity. Also, tombusvirus RNA replication was poor in cell-free extracts or in isolated ER membranes from yeast with repressed Sec39p expression. The tombusvirus p33 replication protein was mislocalized to the ER when Sec39p was depleted in yeast. Overall, Sec39p is the first peroxisomal biogenesis protein characterized that is critical for tombusvirus replication in yeast and plants.

Introduction

Replication of (+)-strand RNA viruses requires the assembly of the membrane-bound viral replicase complexes (VRCs) and replication organelles (Belov and van Kuppeveld, 2012; Huang et al., 2012; Mine and Okuno, 2012; Nagy and Pogany, 2012; Shulla and Randall, 2012). The VRCs consist of viral-coded replication proteins, the viral RNA(s) and subverted host proteins (Nagy and Pogany, 2012; Nagy and Richardson, 2012). The emerging picture with several model (+)-strand RNA viruses is that the assembly of the VRCs within the infected cells is a complex, highly regulated process assisted by an unknown number of factors. Another level of complexity is the subversion of selected subcellular membranes for the VRC assembly. It is currently poorly understood how given subcellular compartments are subverted by different (+)-strand RNA viruses. It seems that different viruses could subvert or modify different subcellular compartments, such as endoplasmic reticulum (ER), mitochondria, peroxisomes; lysosomes/tonoplasts, chloroplasts (for plant RNA viruses) or plasma membrane. In spite of differences in subcellular locations, RNA viruses must manipulate cellular membrane metabolism to build membrane-bound VRCs and replication organelles (Belov and van Kuppeveld, 2012; de Castro et al., 2013; den Boon et al., 2010; Salonen et al., 2005; Syed et al., 2010). These viral-induced structures likely show unique protein and lipid composition. Importantly, these subcellular compartments could be target for emerging antiviral technologies.

Tomato bushy stunt virus (TBSV) and other tombusviruses are fascinating model systems to unravel the mechanism of (+)RNA virus replication and RNA recombination (Nagy, 2008, 2011; Nagy and Pogany, 2010). The roles of the two viral replication proteins, termed p33 and $p92^{pol}$, and several recruited host proteins have been studied in some detail (Li and Nagy, 2011; Nagy et al., 2012; Nagy and Pogany, 2012). It seems that TBSV forms spherule-like structures (vesicles with narrow openings towards the cytosol) utilizing peroxisomal membranes in plants and yeast, a model host (Barajas et al., 2009a; McCartney et al., 2005). Pex19p cytosolic shuttle protein binds to the tombusvirus p33 and $p92^{pol}$ and facilitates their localization to the peroxisomal membranes (Pathak et al., 2008). Interestingly, TBSV replication can efficiently switch to the ER membranes in the absence of peroxisomes (Jonczyk et al., 2007; Rubino et al., 2007). Indeed, systematic genome-wide screens in yeast have not yet revealed major roles for the best-known peroxisomal biogenesis proteins, such as the various pex proteins, in TBSV replication (Jiang et al., 2006; Nagy and Pogany, 2010; Panavas et al., 2005b; Shah Nawaz-Ul-Rehman

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et al., 2012, 2013). Also, TBSV could utilize the isolated ER membranes efficiently for RNA replication *in vitro*, suggesting that the ER membranes are suitable to assemble the TBSV replicase (Xu et al., 2012). Based on these observations, TBSV seems to be able to use both peroxisome and ER-associated factors to assemble the VRCs efficiently.

A previous screen based on temperature-sensitive (ts) mutant library of essential yeast genes identified Sec39p vesicle-mediated transport protein needed for TBSV replication (Shah Nawaz-Ul-Rehman et al., 2013). The Sec39-2^{ts} mutant protein supported TBSV replication poorly at semi-permissive temperature, suggesting that Sec39p might be involved as a stimulatory host factor in TBSV replication. Sec39p is known to be part of an ER localized tethering complex with Dsl1p that interacts with the SNARE (soluble NSF attachment protein receptor) proteins Use1p, Ufe1p and Sec20p. This complex affects the retrograde transport from the Golgi-to-ER and protein secretion (Kraynack et al., 2005; Meiringer et al., 2011; Ren et al., 2009; Spang, 2012). Another important function of Sec39p, together with Sec20p and Dsl1p, is to affect the ER-dependent peroxisome biogenesis and peroxisome protein distribution (Perry et al., 2009). Interestingly, the peroxisomal proteins are mislocalized either to the cytosol or the ER when *SEC*39 expression is down-regulated. Pex3p peroxisome biogenesis protein is mislocalized to the ER and ER-like tubular structures when the expression of Sec39p is repressed, demonstrating that this vesicular transport protein plays a major role in peroxisome biogenesis (Perry et al., 2009). The *Arabidopsis* and human orthologs of the yeast *SEC*39 have been identified and autosomal recessive mutation of this gene causes severe postnatal growth retardation, facial dysmorphism with senile face, small hands and feet, though normal intelligence (Maksimova et al., 2010).

Based on the role of Sec39p in peroxisome biogenesis and TBSV replication in yeast, in this paper, we tested if repression of Sec39p expression affected TBSV replication. We observed that the yeast Sec39p vesicular transport protein was important for TBSV replication in yeast. We document reduction in RNA replication and viral replicase activity in yeast with depleted Sec39p level. Also, we show that the cell-free extract and the ER membrane isolated from yeast with down-regulated Sec39p level supported greatly reduced tombusvirus replication *in vitro*. We also present evidence for the re-localization of the tombusviral p33 replication protein to the ER membrane in Sec39p-depleted yeast cells. Moreover, silencing of the plant *SEC*39-like gene greatly interferes with tombusvirus replication in plants, thus supporting the role of this

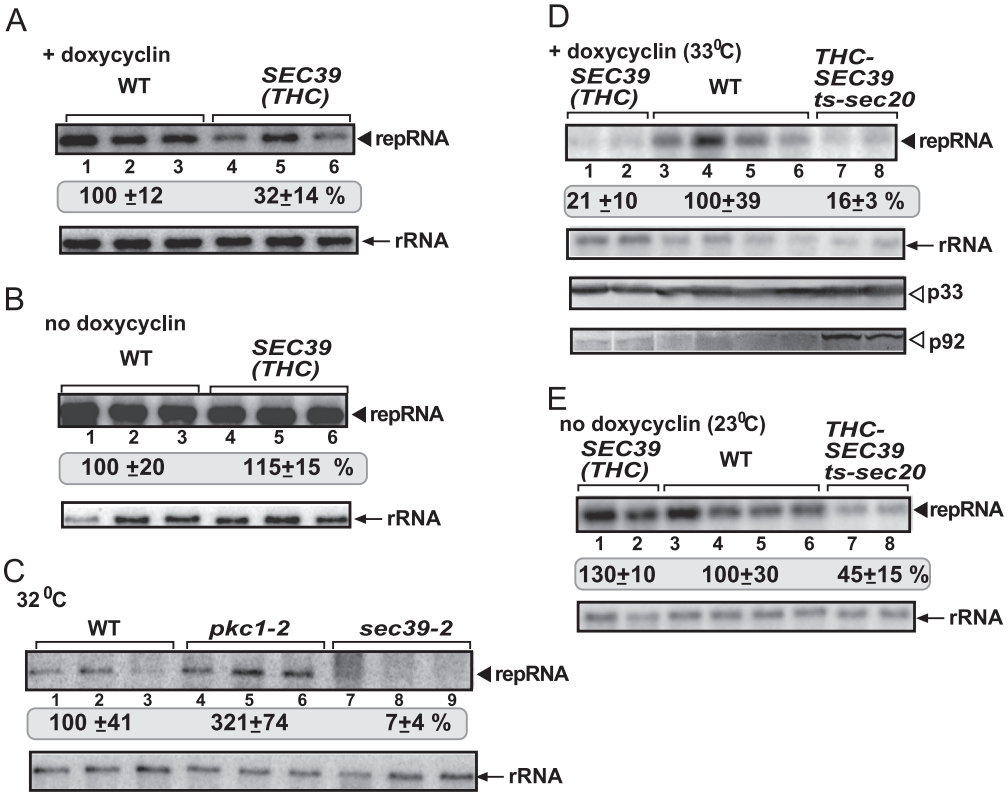

Fig. 1. Repression of *SEC*39 vesicular transport gene inhibits TBSV repRNA accumulation in yeast. (A) Northern blot analysis of TBSV repRNA using a 3′ end specific probe shows the reduced accumulation of repRNA in THC-SEC39 yeast strain. Viral proteins His$_6$-p33 and His$_6$-p92 were expressed from plasmids from the copper-inducible *CUP*1 promoter, while DI-72(+) repRNA was expressed from the galactose-inducible *GAL*1 promoter. TBSV replication was induced by growing yeast cells in media containing 2% galactose (also 2% raffinose as a carbon source) and 50 μM $CuSO_4$ at 23 °C for 36 h, while the expression of *SEC*39 was repressed by doxycycline. Northern blot with 18S ribosomal RNA specific probe was used as a loading control. (B) Northern blot analysis of TBSV repRNA accumulation as in panel A, except the expression of *SEC*39 was not repressed by doxycycline. (C) Northern blot analysis of TBSV repRNA shows reduced accumulation of repRNA in *sec*39-2^{ts} yeast strain grown under semi-permissive temperature. Yeast cells were pre-grown in SC LH$^-$ media containing BCS at 23 °C for 9 h and then for 3 h at 33 °C. The expression of viral proteins His$_6$-p33 and His$_6$-p92 was induced by addition of $CuSO_4$ for 6 h, followed by incubation of yeasts in glucose media containing doxycycline and BCS at 33 °C. WT yeast was used as a control, while the previously published temperature-sensitive *pkc*1-2 is shown as a positive control. Northern blot with 18S ribosomal RNA specific probe was used as a loading control. (D) The combined repression of *SEC*39 expression and partial inactivation of *sec*20^{ts} at semi-permissive temperature greatly inhibits TBSV repRNA accumulation in yeast. Top image: Northern blot analysis of TBSV repRNA accumulation in THC-SEC39/*sec*20^{ts} yeast strain grown at semi-permissive temperature (33 °C) in the presence of doxycycline. Yeasts were pre-grown in glucose media containing doxycycline and BCS at 23 °C for 9 h and then at 33 °C for 3 h. The viral protein expression was induced by addition of $CuSO_4$ for 6 h, followed by incubation of yeasts in glucose media containing doxycycline and BCS at 33 °C. Bottom images: Western blot analysis of the level of His$_6$-p33 and His$_6$-p92 with anti-His antibody in wt and THC-SEC39/*sec*20^{ts} yeast strain. (E) The same as in panel D, except the yeast cells were grown at 23 °C in media in the absence of doxycycline.

host protein in tombusvirus replication. Altogether, we demonstrate a critical role for Sec39p in TBSV replication and propose that Sec39p might be involved in organization of membranous microdomains that are hijacked by TBSV.

Results

Down-regulation of ER-associated Sec39p vesicular transport protein in yeast inhibits TBSV RNA accumulation

To explore the importance of ER-resident Sec39p vesicular transport protein in TBSV replication in yeast, we repressed the transcription of a single copy *SEC*39 from the regulatable TET promoter in THC-SEC39 yeast by addition of doxycycline to the growth media (Mnaimneh et al., 2004). Under this condition, Sec39p is down regulated below detection level by Western blotting (Perry et al., 2009). Northern blot analysis of total RNA from THC-SEC39 yeast (under repressive condition) revealed ~3-fold reduction of TBSV RNA level (Fig. 1A, lanes 4–6 *versus* 1–3). The THC-SEC39 strain supported wt level replication in the absence of doxycycline (not repressed condition) (Fig. 1B, lanes 4–6). Similarly, repression of Sec39p expression decreased TBSV RNA replication by ~5-fold at a higher temperature (33 °C) (Fig. 1D, lanes 1–2), without affecting p33 and p92 levels. Thus, these data suggest that Sec39p vesicular transport protein is critical for TBSV replication in yeast.

To further demonstrate the role of Sec39p functions in TBSV replication, we used a temperature-sensitive Sec39p mutant at a semi-permissive temperature. As observed before in a high-throughput screen (Shah Nawaz-Ul-Rehman et al., 2013), sec39-2^{ts} supported less than 10% TBSV repRNA accumulation in yeast at a semi-permissive temperature (Fig. 1C, lanes 7–9 *versus* 1–3). In contrast, yeast carrying a ts mutant of Pkc1p supported high level of TBSV repRNA accumulation (Fig. 1C), thus demonstrating that TBSV replication could take place at elevated temperature. As we have shown previously, Protein kinase C (Pkc1p) is a cellular restriction factor that phosphorylates the TBSV p33 and p92 replication proteins rendering them inactive to bind to the viral RNA (Shah Nawaz-Ul-Rehman et al., 2013).

To further test the involvement of Sec39p vesicular transport protein in TBSV replication, we generated a double mutant yeast strain, carrying THC-SEC39 and *sec*20^{ts}. This double mutant yeast strain supported poor TBSV replication, resulting in ~5-fold reduction in TBSV repRNA level under repressive transcription and at semi-permissive temperature (Fig. 1D). The accumulation level of p33 and p92^{pol} increased in THC-SEC39/*sec*20^{ts} yeast (Fig. 1D, lanes 7–8), suggesting that expression level of p33 and p92^{pol} is unlikely the reason for the dramatic decrease of TBSV repRNA accumulation in this yeast strain under repressive transcription and at a semi-permissive temperature. The double mutant showed lesser effect on TBSV replication under non-repressive transcription and at permissive temperature (Fig. 1E, lanes 7–8). Altogether, these data suggest that the ER-associated vesicular transport protein Sec39p, which is involved in peroxisome membrane organization, is critical for TBSV replication in yeast.

Purified TBSV replicase from THC-SEC39/sec20^{ts} yeast has low activity in vitro

The reduced level of TBSV repRNA accumulation from THC-SEC39 or the double mutant THC-SEC39/*sec*20^{ts} yeasts might be due to inefficient tombusvirus replicase assembly. To test this possibility, we affinity-purified TBSV replicase from THC-SEC39/*sec*20^{ts} yeast grown under repressive condition and at semi-permissive temperature (Fig. 2A). The solubilized and affinity-purified tombusvirus replicase can efficiently utilize externally added (−) repRNA to make complementary RNA products (Panaviene et al., 2005; Panaviene et al., 2004). Using (−)RNA as a template (Panavas et al., 2002), we found that the *in vitro* activity of the tombusvirus replicase obtained from THC-SEC39/*sec*20^{ts} yeast decreased by ~70% when compared to the replicase from wt yeast (Fig. 2B lane 2 *versus* lane 1). These two tombusvirus replicase preparations contained comparable amounts of p33 (Fig. 2B), excluding that the observed differences in activity in these preparations are due to differences in replication protein level. Based on these data, we suggest that either the assembly or the activity of the tombusvirus replicase is hindered in THC-SEC39/*sec*20^{ts} yeast.

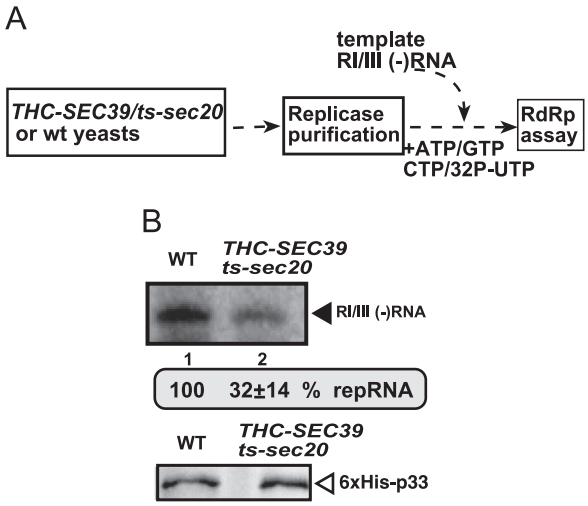

Fig. 2. Reduced activity of the affinity-purified TBSV replicase derived from THC-SEC39/*sec*20^{ts} yeast strain. (A) Scheme of the replicase purification and *in vitro* assay. TBSV replicase was His-affinity purified via His$_6$-tagged p33 and p92 viral replication proteins from wt and THC-SEC39/*sec*20^{ts} yeast strains expressing His$_6$-tagged p33 and p92^{pol} from the *CUP*1 promoter and DI-72 repRNA from the *ADH*1 promoter in the presence of doxycycline at 33 °C for 6 h before purification. Yeast cells were pre-grown for 16 h at 33 °C in the presence of doxycycline. Then RI/III (−)RNA [which contains the cPR promoter for (+)-strand synthesis and the RIII replication enhancer region] was used to program the purified replicase to produce ^{32}P-labeled complementary RNA product. The *in vitro* replication was conducted for 4 h. (B) Top image: The RNA products of the above replicase assay were analyzed on denaturing PAGE. Bottom image: Western blot analysis to estimate the amount of His$_6$-tagged p33 in the His-affinity purified TBSV replicase using anti-His antibody.

Reduced TBSV replication in cell free extract from THC-SEC39/sec20^{ts} yeast is due to membrane-associated factors

Since the ER-associated vesicular transport proteins Sec39p is involved in peroxisome membrane organization, it is possible that Sec39p affects the TBSV replicase due to altering ER and/or peroxisomal membranes. To address this question, we took advantage of an *in vitro* tombusvirus replication assay based on cell-free yeast extracts (CFE) (Pogany et al., 2008). The yeast CFEs can be programmed with TBSV (+)repRNA and recombinant tombusvirus p33 and p92^{pol} purified from *E. coli*, which leads to *in vitro* assembly of active replicase complex. Importantly, the CFE supports a single full cycle of TBSV replication (Pogany et al., 2008). We found that CFE prepared from THC-SEC39/*sec*20^{ts} yeast supported TBSV repRNA replication by ~4-fold less efficiently than CFE from wt yeast (Fig. 3B, lanes 9–10 *versus* 3–4).

To test if the reduced TBSV replication in the CFE is based on the membrane (ME) or soluble (cytosolic) fractions of the THC-SEC39/*sec*20^{ts} yeast, we obtained the membrane and the cytosolic

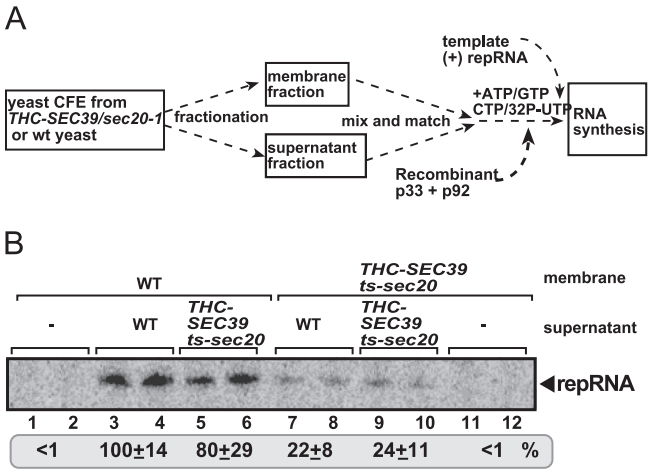

Fig. 3. The membrane fraction of the CFE derived from THC-SEC39/*sec*20^{ts} yeast is critical for TBSV repRNA replication *in vitro*. (A) The scheme of the experiments. The supernatant (soluble fraction) and the membrane fractions (ME) of CFEs from THC-SEC39/*sec*20^{ts} or WT yeasts were separated by high-speed centrifugation (35,000*g*) and, then, the various fractions were mixed as indicated. Yeast were cultured in the presence of doxycycline for 8 h at 29 °C and then for 1 h at 37 °C before production of CFE preparations. Purified recombinant MBP-p33, MBP-p92^{pol} and T7 transcripts of TBSV DI-72 (+)repRNA were added to yeast CFE fractions together with isotope labeled and unlabeled nucleotides to initiate viral replication. (B) The ^{32}P-labeled repRNA products were analyzed on denaturing PAGE. Note that lanes 3–4 show repRNA replication in the WT CFE (*i.e.*, both the membrane and soluble fractions were from WT yeast), while lanes 9–10 show replication in THC-SEC39/*sec*20^{ts} CFE.

fractions of wt or THC-SEC39/*sec*20^{ts} yeasts with high-speed centrifugation prior to the replication assay (Fig. 3A). Mixing the ME fraction from THC-SEC39/*sec*20^{ts} yeast with the cytosolic fraction from wt CFE resulted in ~4-fold reduced TBSV replication (Fig. 3B, lanes 7–8). On the contrary, mixing the wt ME fraction with the soluble fraction from THC-SEC39/*sec*20^{ts} yeast only led to slight reduction in *in vitro* TBSV replication (Fig. 3B, lanes 5–6). As expected, the ME fractions alone (in the absence of the soluble fraction) did not support tombusvirus replication (Fig. 3B, lanes 1–2 and 11–12). Based on these results, we propose that the membrane fraction of CFE prepared from THC-SEC39/*sec*20^{ts} yeast contained host factors that are less suitable to support TBSV replication than host factors present in the ME fraction of wt yeast cells. On the contrary, soluble fraction from THC-SEC39/*sec*20^{ts} yeast seems to be compatible with TBSV repRNA replication *in vitro*.

Requirement for functional Sec39p for peroxisomal localization of tombusvirus replication protein in yeast

The major peroxisome biogenesis protein Pex3p is mislocalized to the ER membrane and ER tubular structures in THC-SEC39 yeast under repressive condition (Perry et al., 2009), while it is mostly present in the peroxisomal membrane in wt yeast. To test if p33 replication protein is also mislocalized in THC-SEC39 yeast under repressive condition, we followed the localization of GFP-tagged p33 and the RFP-tagged cellular Pho86p (an ER marker protein) (Panavas et al., 2005a). Interestingly, GFP-p33 co-localized with RFP-Pho86p in THC-SEC39 yeast under repressive condition (Fig. 4A). In contrast, GFP-p33 showed punctate structures that did not extensively co-localize with the ER marker protein in THC-SEC39 yeast under non-repressive condition (Fig. 4B) or in wt yeast (Fig. 4C). Similarly, CFP-p33 co-localized with Pho86-YFP ER marker protein in THC-SEC39/*sec*20^{ts} yeast grown under repressive condition and at semi-permissive temperature (Fig. 4F), while CFP-p33 only poorly co-localized with Pho86-YFP ER marker protein in WT yeast (Fig. 4G). These data support that p33 is mislocalized to the ER in yeast with down-regulated Sec39p level.

To see if p33 is co-localized with the Pex3p peroxisome biogenesis protein, we co-expressed RFP-p33 and Pex3-GFP in yeast strains. We found co-localization of RFP-p33 and Pex3-GFP in THC-SEC39 yeast under repressive condition (Fig. 4D). Instead of the punctate appearance, which is characteristic for peroxisome localized proteins (which is the case for both Pex3p and p33 in WT yeast, Fig. 4E) (Perry et al., 2009), both Pex3p and p33 were mostly part of elongated structures in THC-SEC39 yeast under repressive condition (Fig. 4D). These structures are likely part of the ER membrane and ER tubular structures as shown previously for Pex3p in THC-SEC39 yeast under repressive condition (Perry et al., 2009).

To further demonstrate the mislocalization of p33 in yeast cells deficient in Sec39p functions, we used a temperature-sensitive Sec39p mutant at a semi-permissive temperature. This sec39-2^{ts} mutant yeast also showed robust mislocalization of CFP-p33 to the ER membranes at a semi-permissive temperature (Fig. 4H). In contrast, p33 replication protein formed mostly punctate structures in wt yeast that were proximal to ER membranes (Fig. 4I). Only a fraction of p33 was not part of punctate structures in wt yeast and these molecules seemed to be mislocalized to the ER at elevated temperature (Fig. 4I). Altogether, cellular localization data suggest that the p33 replication protein requires functional Sec39p for correct localization to the peroxisomes. Also, p33 behaves similar to the cellular Pex3p and both proteins end up in ER-derived structures in yeast with reduced Sec39p function.

Reduced in vitro TBSV replication in microsomal preparation from THC-SEC39/sec20^{ts} yeast

To better understand the role of the ER membranes in tombusvirus replication in THC-SEC39/*sec*20^{ts} yeast (under repressive condition), we isolated microsomal fraction (representing the ER membrane), followed by *in vitro* replication assay with purified recombinant tombusvirus replication proteins (Fig. 5A). Interestingly, we found that the microsomal preparation from THC-SEC39/*sec*20^{ts} yeast supported repRNA replication ~11-fold less efficiently than similar prep from WT yeast (Fig. 5B, lanes 4–6 *versus* 1–3). These microsomal preparations contained similar amount of Sec61p ER marker protein (Fig. 5B). We have not tried to use purified peroxisomes from THC-SEC39/*sec*20^{ts} yeast, because WT and especially THC-SEC39 yeasts contain only small amount of peroxisomes (Perry et al., 2009) and the isolated peroxisomes (induced by oleic-acid treatment) from WT yeast did not support TBSV replication *in vitro* (Xu et al., 2012). Overall, these data suggest that the tombusvirus replicase assembled in the isolated ER membrane from THC-SEC39/*sec*20^{ts} yeast can support TBSV replication only poorly.

The tombusvirus p33 replication protein interacts with Sec39p

One of the possibilities for the requirement of Sec39p for tombusvirus replication in yeast is that Sec39p might facilitate the proper organization and/or transport of p33 inside the cell. This putative function could be facilitated by interaction between these molecules. Therefore, we tested molecular interaction between p33 and Sec39p in yeast based on split-ubiquitin membrane yeast two-hybrid assay. Briefly, the split-ubiquitin assay utilizes the ability of N-terminal and C-terminal halves of ubiquitin to reconstitute a functional ubiquitin (Fetchko et al., 2003; Fetchko and Stagljar, 2004). The two halves of ubiquitin, which are fused separately to the test proteins, are brought to close proximity by the interacting protein domains. This then leads to the cleavage of the reconstituted ubiquitin by endogenous ubiquitin specific proteases, resulting in the release of the transcription factor, which

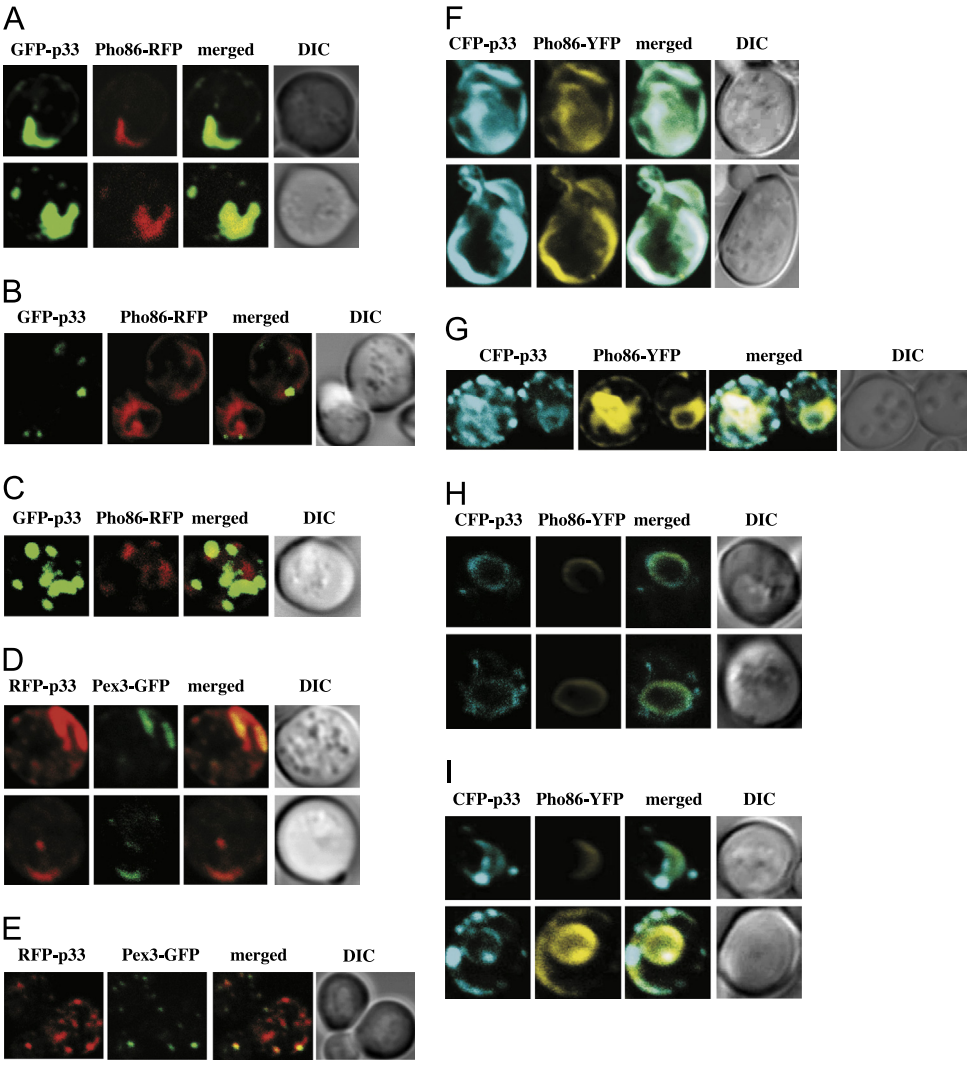

Fig. 4. Altered subcellular localization of p33 in THC-SEC39 yeast under repressive condition. (A) Confocal laser microscopy analysis of subcellular distribution of GFP-tagged p33 in THC-SEC39 yeast. Cells were treated with doxycycline to repress Sec39p expression. Pho86-RFP was used as an ER marker. (B) The same as in panel A, except doxycycline was omitted. GFP-p33 forms wt-like punctate distribution (likely peroxisomal distribution) in the vicinity of ER. (C) Peroxisomal distribution of GFP-p33 in wt yeast in the presence of doxycycline. (D) Co-localization of RFP-p33 with GFP-Pex3p in THC-SEC39 yeast under repressive condition (treatment with doxycycline to repress Sec39p expression). Note that both p33 and Pex3p form long, tubule-like structures instead of the characteristic punctate structures when present in the peroxisomes. This suggests that p33 and Pex3p are mislocalized in THC-SEC39 yeast under repressive condition. (E) Confocal laser microscopy analysis shows characteristic punctate structures formed by both p33 and Pex3p, indicative of peroxisomal localization in WT yeast. (F) Co-localization of CFP-p33 with YFP-Pho86p in THC-SEC39/*sec*20^{ts} yeast under repressive condition at semi-permissive temperature (34 °C). (G) CFP-p33 is only poorly co-localized with YFP-Pho86p in wt yeast grown at 34 °C in the presence of doxycycline. (H) Confocal laser microscopy analysis shows ER distribution of CFP-tagged p33 in *sec*39^{ts} yeast strain grown under semi-permissive temperature. Pho86-YFP was used as an ER marker. (I) The same as in panel G, except WT yeast was used. GFP-p33 mostly forms wt-like punctate distribution (likely peroxisomal distribution) in the vicinity of ER in these cells grown at 33 °C.

allows growth on selective media. We found that p33 interacted with Sec39p in this assay (Fig. 6A). However, it seems that Sec39p interaction with p33 is less robust than the interaction of p33 with Ssa1p (Hsp70 chaperone), which is an important component of the tombusvirus replicase complex (Pogany et al., 2008; Serva and Nagy, 2006; Wang et al., 2009a, 2009b).

To further confirm the interaction, we performed co-purification experiments from yeast co-expressing FLAG-His$_6$-tagged p33 and 6xHA-tagged Sec39p. The yeast strain expressed 6xHA-tagged Sec39p from the natural promoter and from the chromosome in the absence of wt Sec39p. After solubilization of the membrane-fraction, the FLAG-His$_6$-p33 was immobilized to the FLAG column. After elution of the bound proteins from the column, we analyzed if 6xHA-tagged Sec39p host protein was present in the eluted fraction by using anti-HA antibody and Western-blotting (Fig. 6B). Similarly prepared yeast extract containing His$_6$-p33 was the negative control to exclude non-specific binding by 6xHA-tagged Sec39p. This co-purification experiment demonstrated the specific binding of Sec39p to p33 in yeast membranes (Fig. 6B).

Knocking down the level of the plant SEC39 transport protein inhibits TBSV accumulation in Nicotiana benthamiana plants

To confirm that the above findings on the key role of Sec39p in TBSV replication in the yeast model host are also valid in plant hosts, we decided to use *Nicotiana benthamiana,* an experimental host. To inhibit the activity of Sec39p, we used a virus-induced gene silencing (VIGS) approach that resulted in down-regulation of *SEC*39 mRNA (Fig. 7A). Replication of tombusvirus genomic RNA decreased by ~10-fold in the *SEC*39 knockdown plants when compared to the nonsilenced plants (Fig. 7A, lanes 7–12

versus 1–6). These data suggest that Sec39p is also required for TBSV accumulation in plants. Silencing of *SEC*39 also inhibited the growth of the newly emerging leaves, which stayed much smaller than in nonsilenced plants and the apical terminal shoots stopped growing (Fig. 7B).

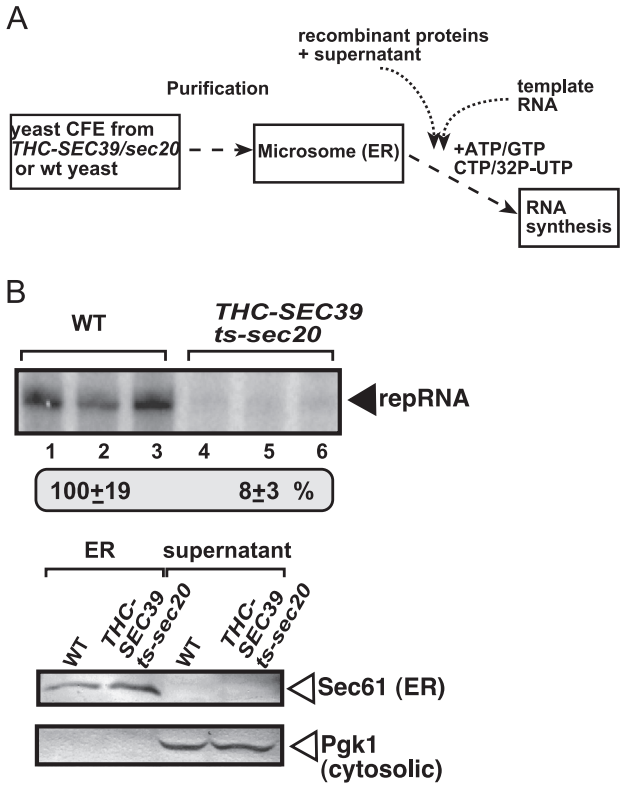

Fig. 5. Poor replication of TBSV repRNA in microsome preparation obtained from THC-SEC39/*sec*20^{ts} strain. (A) Scheme of the replication assay. The isolated ER (microsome) preparations from THC-SEC39/*sec*20^{ts} (grown under repressive condition and at the semi-permissive 33 °C) and wt yeast (cultured using the same condition as the double-mutant yeast strain) were programmed with purified recombinant MBP-p33 and MBP-p92 as well as the TBSV-derived (+)repRNA *in vitro*. (B) Top image: denaturing PAGE analysis of the ^{32}P-labeled repRNA products obtained in the replication assays with the isolated yeast microsome preparations. The synthesized full-length repRNA is pointed at by an arrowhead. The experiment was repeated twice. Bottom images: Western blot analysis of Sec61p (ER marker) and Pgk1p cytosolic marker proteins in the microsome preparation with the help of specific antibodies.

Down-regulation of Sec39p in yeast does not inhibit Flock house virus RNA accumulation

To test whether the functional Sec39p is critical for another RNA virus, we choose Flock house virus, an insect virus, which replicates using the mitochondrial membrane in yeast (Van Wynsberghe et al., 2007; Venter and Schneemann, 2008) and is not related to TBSV. We found that FHV RNA accumulation increased ~3-fold in THC-SEC39

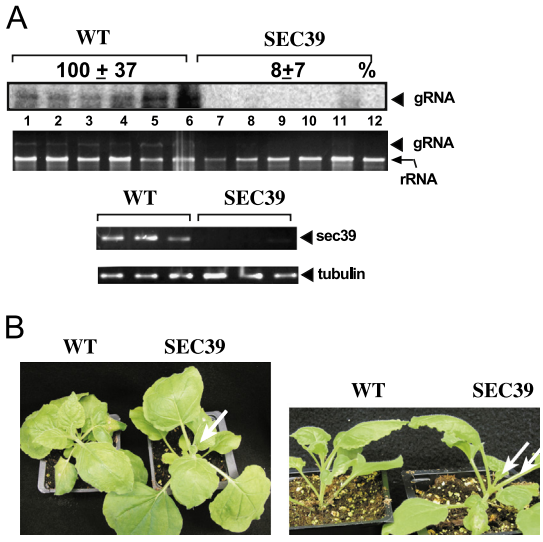

Fig. 7. Knockdown of *SEC*39-like gene inhibits TBSV RNA replication in *N. benthamiana* plants. (A) Top panel: Reduced accumulation of TBSV RNA in the inoculated leaves of *SEC*39 knockdown *N. benthamiana* plants 3 days post-inoculation, based on Northern blot analysis. Inoculation with TBSV gRNA was done 9 days after silencing of *SEC*39 expression by sap inoculation. VIGS was performed via agroinfiltration of *Tobacco rattle virus* (TRV) vectors carrying *SEC*39 sequence or the TRV empty vector (as a control). Second Panel: Ribosomal RNA is shown as a loading control. Note that the genomic TBSV RNA is also visible in the gel. Third panel: RT-PCR analysis of *SEC*39 mRNA level in the silenced and control plants. Fourth panel: RT-PCR analysis of *TUBULIN* mRNA level in the silenced and control plants. (B) Phenotype of the *SEC*39 knockdown *N. benthamiana* plants 9 days after agroinfiltration with the VIGS vectors. Note that silencing of *SEC*39 inhibited the growth of the newly emerging leaves, which stayed much smaller than in nonsilenced plants (indicated by a white arrow).

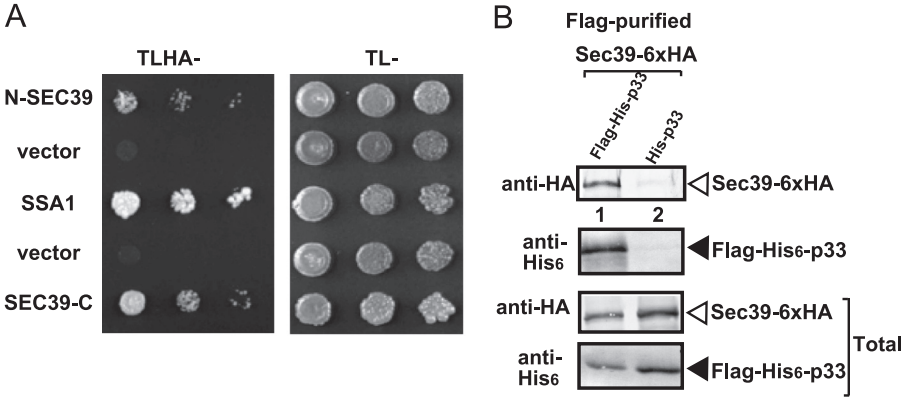

Fig. 6. Interaction between p33 replication protein and the yeast Sec39p protein in the intracellular membrane. (A) The split ubiquitin assay was used to test binding between p33 and Sec39p in yeast. The bait p33 was co-expressed with either N-terminally or C-terminally tagged Sec39p protein. *SSA*1 (HSP70 chaperone) and the empty prey vector (NubG) were used as positive and negative controls, respectively. (B) Co-purification of Sec39p with the p33 replication protein from subcellular membranes. Top panel: Western blot analysis of co-purified HA-tagged cellular Sec39p protein with Flag-affinity purified FLAG/His$_6$-p33. Sec39p was detected with anti-HA antibody, while FLAG/His$_6$-p33 was detected with anti-His antibody (second panel from top). The negative control was His$_6$-tagged p33 purified from yeast extracts using a FLAG-affinity column. Middle bottom panel: Western blot of total HA-Sec39p in the total yeast extract using anti-HA antibody. Bottom panel: Western blot of total FLAG/His$_6$-tagged p33 in the total yeast extract using anti-His antibody.

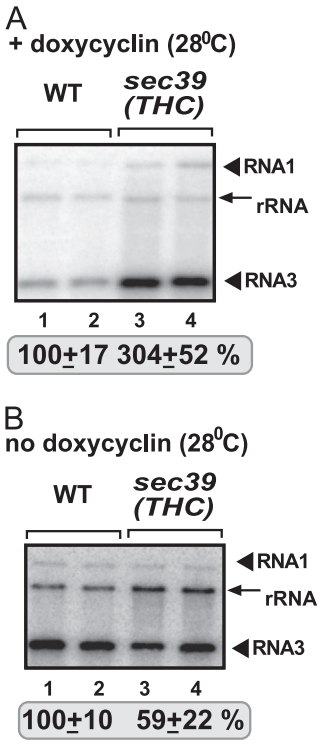

Fig. 8. Repression of *SEC*39 vesicular transport gene does not inhibit the unrelated FHV RNA accumulation in yeast. (A) Northern blot analysis of FHV RNA1 and the subgenomic RNA3 (made from RNA1 during replication by the FHV replicase) using a 3′ end specific probe shows the increased accumulation of RNA3 in THC-SEC39 yeast strain under repressive condition (added doxycycline). The accumulation of RNA3 was quantitated. Northern blot with 18S ribosomal RNA specific probe was used as a loading control. (B) Northern blot analysis of FHV RNA1 and the subgenomic RNA3 accumulation as in panel A, except the expression of *SEC*39 was not repressed by doxycycline. The experiment was repeated three times.

yeast under repressive condition (Fig. 8A), while it accumulated less efficiently in THC-SEC39 yeast under nonrepressive condition (Fig. 8B). Thus, FHV accumulation was affected differently from that of TBSV by the availability of Sec39p in yeast cells. It seems that Sec39p facilitates TBSV replication in a specific manner.

Discussion

TBSV replication seems to be a rather dynamic process in infected cells by mostly taking advantage of peroxisomal membranes or in the absence of peroxisomes, the ER membranes (Jonczyk et al., 2007; McCartney et al., 2005; Panavas et al., 2005a; Xu et al., 2012). TBSV and the related CNV replication proteins bind to Pex19p peroxisomal shuttle protein (McCartney et al., 2005; Pathak et al., 2008), which is involved in delivering ~30 peroxisomal membrane proteins to the peroxisomes (Fagarasanu et al., 2010; Titorenko and Rachubinski, 2009). The interaction between p33 replication protein and Pex19p aids the targeting of p33 replication protein to the peroxisomal membranes (McCartney et al., 2005; Pathak et al., 2008). However, the p33: Pex19p interaction is not essential for TBSV replication, which can take place in the absence of Pex19p and peroxisomal membranes by utilizing the ER membranes in yeast (Jonczyk et al., 2007). Also, Pex3p peroxisome biogenesis protein, the binding partner of Pex19p during cargo delivery to the peroxisome membranes, does not seem to be critical for TBSV replication in yeast (Panavas et al., 2005b). How p33 is targeted to the ER membrane (in the absence of peroxisomes) is currently unknown.

The current work has unraveled a critical role for Sec39p vesicular transport protein in TBSV replication. Down-regulation of Sec39p in yeast (Fig. 1A) or a *sec*39^{ts} mutant at semi-permissive temperature (Fig. 1C) led to: (i) greatly reduced level of TBSV RNA replication; (ii) low tombusvirus replicase activity (Fig. 2); and (iii) poor replication in a CFE assay (Fig. 3). In addition, the isolated ER membranes from yeast (THC-SEC39/*sec*20^{ts}) showed greatly reduced ability to support TBSV replication *in vitro* (Fig. 5). All these data support a model that Sec39p vesicular transport protein plays a critical role in TBSV replication. This role might be direct since we found interaction between the membrane-bound p33 and Sec39p as discussed below. Importantly, the possible harmful effect of down-regulation of essential Sec39p vesicular transport protein on retrograde transport between the Golgi and the ER on the general status of the cell, and thus, indirectly on TBSV replication, is unlikely since the mitochondria membrane-based FHV replication was not inhibited by down-regulation of Sec39p level (Fig. 8). Therefore, we suggest that the inhibitory effect of down-regulation of Sec39p on TBSV replication in yeast is likely specific against this virus that replicates in peroxisome/ER membranes. In addition, the Sec39-like plant protein likely plays a similar role to the yeast Sec39p in TBSV replication, since silencing the expression of *SEC*39-like gene in *N. benthamiana* decreased tombusviral RNA accumulation (Fig. 7).

Subcellular localization studies with p33 replication protein revealed that p33 mislocalized to the ER in THC-SEC39/*sec*20^{ts} yeast under restrictive condition (Fig. 4). This was expected since peroxisome biogenesis is hindered under this condition due to the essential roles of Sec39p in peroxisome membrane organization (Perry et al., 2009). The cellular distribution of p33 looked similar to Pex3p, which is known to be mistargeted to ER membranes and ER tubular structures in yeast with low Sec39p level (Perry et al., 2009). It was surprising, however, that the activity of the tombusvirus replicase decreased in THC-SEC39/*sec*20^{ts} yeast under restrictive condition, although it is known that the wt yeast ER membrane could support efficient TBSV replication (Jonczyk et al., 2007; Pathak et al., 2008). Based on these observations, we suggest that Sec39p cellular protein plays a direct role in TBSV replication by, for example, anchoring the TBSV replication proteins or the replicase to the ER membrane. Accordingly, we found interaction between the membrane-bound p33 and Sec39p (Fig. 6). Alternatively, Sec39p and associated cellular factors might play indirect roles. For example, Sec39p might be involved in affecting the local lipid composition within the ER membrane (affecting lipid rafts in ER). Indeed, cellular lipids, such as phospholipids and sterols play critical roles in TBSV replication (Sharma et al., 2010, 2011). Moreover, Sec39p could be involved in the regulation of the distribution of ER resident cellular proteins that directly affect the TBSV replicase in the ER membrane. As an example, Sec39p or associated cellular factors could be involved in formation of specific subdomains, called peroxisomal ER or peroxisome template (Titorenko and Rachubinski, 2009), within the general ER membrane, which are suitable for the assembly of the tombusvirus replicase complex. Down-regulation of Sec39p might prevent the formation of these peroxisome-like areas within the vast ER membranes, which could greatly inhibit the assembly of the tombusvirus replicase in those subcompartments or microdomains. Future experiments will address these alternative models. Altogether, identification of the critical function of Sec39p in this work opens up the opportunity to study the role of peroxisome biogenesis and ER microdomains in TBSV replication.

Materials and methods

Yeast strains and expression plasmids

Saccharomyces cerevisiae strain BY4741 (*MATa his*3Δ1 *leu*2Δ0 *met*15Δ0 *ura*3Δ0), THC-SEC39 and R1158 (wt) (Mnaimneh et al.,

2004) were obtained from Open Biosystems. The temperature-sensitive (ts) yeast strains *sec*39-2^{ts} and *sec*20-1^{ts} were of generous gift from C. Boone (U. Toronto). The THC-SEC39 and wt yeast strains with GFP-tagged *PEX*3 inserted into the chromosome were created in the Rachubinsky lab (Perry et al., 2009). pGAD-ADH1-Pho86-RFP plasmid (*leu*2 selection) was generated previously (Panavas et al., 2005a).

pESC-CUP1-GFP-p33 (*his*3 selection) was created by PCR through amplifying GFP-p33 cassette with primers #4568F (GGCAGATCTGGTAAAGGAGAAGAACTTTTCACT) and #2182R (GTC-GTCGACTTAATCGATGCTAGCCCATGGCCCGGG-*TTTCACACCAAGGG-ACTC*) on the template plasmid pYES-GAL1-GFP-p33 (Wang et al., 2009a). The primers contain BglII and SalI restriction sites, respectively. After BglII and SalI digestion, the PCR product was cloned into pESC-CUP1 plasmid (Pogany and Nagy, 2008) digested with BamHI and XhoI. The insertion was confirmed with *CUP*1 promoter specific primer #2753F (GCCGCTAGCGACATTTGGGCGC-TATACGT) and p33 specific #992bR (GAGCTGCAGCTATTTCACAC-CAAGGGA). pGAD-CUP1-RFP-p33 (*leu*2 selection) was created by PCR-amplifying RFP-p33 cassette with primers #2691F (CGGA-GATCTATGGCCTCCTCCGAGGAC) and #2182R on the template plasmid pGAD-ADH1-RFP-p33 (Barajas et al., 2009b). The primers contain BglII and SalI restriction sites, respectively. After BglII and SalI digestion, the PCR product was cloned into pGAD-CUP1-p92 (Serviene et al., 2006) by digesting the plasmid with BamHI and XhoI restriction enzymes and replacing p92 ORF with RFP-p33.

The double mutant THC-SEC39/ts-sec20-1 yeast strain was created by transforming THC-SEC39 with the PCR amplified sequence of ts-*sec*20-1 gene as described for the creation of the temperature sensitive mutant yeast library (Li et al., 2011) with some modifications. Briefly, hygromycin resistance marker gene was PCR-amplified with primers #5069F (CGTACGCTGCAGGTC-GAC) and #5070R (TAAGTATATGCGCTTATTATGTT-ATATAGATGT-TAAAATTAATCGATGAATTCGAGCTCG) using the Euroscarf plasmid pFA6a-hphNT1 (Janke et al., 2004) linearized with SpeI. The above reverse primer included 45 nucleotides matching the 3′UTR sequence of the wt *SEC*20 gene. For the PCR-amplification of ts-*sec*20-1 gene on the purified yeast genomic DNA of ts-*sec*20-1 strain, primers #5073F (carrying sequences upstream of the *SEC*20 gene) (GAAAGCACGTCTTTACCAGTGACTT) and #5071R (carrying sequences downstream of the *SEC*20 gene and a short overlap with primer #5069) (GGTCGACCTGCAGCGTACGTATAAGCTATACC-TAACGCATGTGAAG) were used. Both PCR products were purified and co-transformed into the THC-SEC39 yeast strains. Correct integration of the hygromycin marker gene relative to ts-*sec*20-1 was confirmed with primers #5113F (CCTAGGTCTAGTTAAAAGC-TATGCAGGCTC) and #2215R (CTGCAGCGAGGAGCCGTAAT) and also with primers #5115F (ACCTTTAGCAAACGTTGGAA) and #2215R. #5113F primer targets the 3′ region of the *SEC*20 gene and #5115F targets an upstream sequence to *SEC*20. Temperature sensitivity of the resulting double-mutant strains was confirmed by spotting serial dilutions on YPD-hygromycin plates and placed them both into 29 °C and 37 °C incubators. We also co-transformed the wt BY4741 strain with the PCR products made on ts-*sec*20-1 template and hygromycin resistance cassette and serial dilutions of the transformed colonies were spotted on YPD-hygromycin plates and we placed them both into 29 °C and 37 °C incubators. The yeast growth was restricted at 37 °C, confirming the temperature sensitivity of the obtained yeast strains.

TBSV repRNA and FHV RNA accumulation assays in yeast

Yeast strains R1158 (wt), and THC-SEC39 were transformed with pESC-GAL1-6xHisp33/GAL10-DI-72 (*his*3 selection) and pGAD-CUP1-6xHisp92 (leu2 selection) (Serviene et al., 2006). Cells were grown overnight in Sc LH$^-$ media containing 2% galactose and 2% raffinose at 23 °C in the presence of 50 μM $CuSO_4$ and doxycycline (20 μg/ml) to down-regulate the expression of the relevant host protein, for 36 h at 29 °C. Then, total RNA was extracted and TBSV repRNA accumulation was detected by Northern blot analysis. The accumulation of viral RNA levels were normalized based on rRNA levels in the total RNA samples.

As galactose media is less suitable for viral RNA accumulation studies in the yeast ts strains than glucose containing media, THC-SEC39, and THC-SEC39/ts-sec20 and wt strains were transformed with pGBK-CUP1-6xHisp33/ADH1-DI-72 (*his*3 selection) together with pGAD-CUP1-6xHisp92 (*leu*2 selection) (Mendu et al., 2010). Cells were grown overnight in SC LH$^-$ glucose media at 23 °C in the presence of 100 μM bathocuproine disulphonate (BCS) copper chelator. Then, to down-regulate the expression of the relevant host protein, doxycycline (20 μg/ml) was added to the media for 9 h and then cultures were placed at 33 °C for 3 h before inducing TBSV replication. Then, cells were centrifuged and washed thoroughly with SC LH$^-$ glucose media containing 20 μg/ml doxycycline and 50 μM $CuSO_4$ to induce viral protein expression. Cells were grown at 33 °C for 6 h, then $CuSO_4$ was replaced with BCS after rigorous washing of the cells with glucose media. Yeasts were kept at 33 °C for additional 18 h. Then, total RNA was extracted, phenol/chloroform precipitated and the TBSV repRNA accumulation was detected by Northern blot analysis with 3′ specific P^{32}-labeled probe to the repRNA (Panavas and Nagy, 2003).

In the case of the FHV assay, THC-SEC39 and R1158 yeast strains were transformed with pESC-CUP1-FHV-RNA1-TRSV$_{Rz}$ (*his*3 selection) and the yeasts were grown as above, except at 29 °C. FHV RNA accumulation was tested as described (Kovalev et al., 2012; Pogany et al., 2010).

In vitro TBSV replication assay based on yeast cell-free (CFE) extract

CFEs from THC-WT and THC-SEC39/ts-sec20-1 mutant strains were prepared and the replication assay was carried out as described earlier (Pogany et al., 2008) with the following modifications. The above yeast strains were pre-grown in YPD with increasing volume from 2 to 20 ml. Then, the total cultures were increased to 200 ml and OD_{600} was adjusted to 0.4, and doxycycline was added to a final concentration of 20 μg/ml and cells were grown for additional 8 h. Then, temperature was increased to 37 °C (the restrictive temperature for ts-sec20-1 mutation) for 2 h, followed by harvesting the cells for CFE preparation. Membrane and soluble fractions were separated by centrifugation at 35,000*g* and then mixed in various combinations as described in the figure legend.

Replicase purification and in vitro TBSV replication assay

Yeast strain R1158 and THC-SEC39/ts-sec20-1 mutant strain were transformed with pGBK-CUP1-6xHisp33/ADH1-DI-72 together with pGAD-CUP1-6xHisp92 (*leu*2 selection). Cells were grown in SC LH$^-$ glucose containing 100 μM BCS at 23 °C, and the culture volume was gradually increased from 2 ml to 20 ml. At the end, the culture volume was increased to 200 ml, and the OD_{600} was set to 0.4, followed by the addition of doxycycline at a concentration of 20 μg/ml and the incubation temperature was increased to 32 °C for 12 h. Then, BCS was washed out and replaced with 50 μM $CuSO_4$ to induce the expression of p33 and p92^{pol} viral proteins. Yeasts were grown for an additional 6 h. For purification of the viral replicase, yeast membranes were solubilized with detergent (Li et al., 2008), followed by affinity purification via His-tagged p33 using Ni-NTA agarose affinity chromatography matrix as described earlier (Li et al., 2008; Panaviene et al., 2004). The activity of the purified replicase was tested by programming the replicase with 0.5 μg/μl repRNA

(RI/RIII(−) RNA). The *in vitro* assays were performed at 25 °C for 3 h in the presence of P^{32}-labeled UTP as described (Panaviene et al., 2004). Then, the RNA was phenol/chloroform purified, precipitated by isopropanol-ammonium acetate and analyzed in 5% denaturing PAGE containing 8M UREA (Panaviene et al., 2004).

Microsomal membrane preparation and in vitro replicase activity assay

Yeast strains THC-SEC39/tsSEC20-1 and R1158 were pre-grown in YPD overnight, then the culture volume was increased from 2 ml to 20 ml and cultures were grown for additional 8 h at 29 °C. Then, culture volume was increased to 200 ml, and doxycycline was added at the concentration of 20 μg/ml and the incubation temperature was shifted to 32 °C for 24 h. The yeast cultures were gradually diluted to keep $OD_{600} < 2.0$. The microsome (representing ER membranes) preparation was obtained by making spheroplasts as described (Wang et al., 2009a). The ER membrane was isolated with sucrose gradient centrifugation at 100,000*g* and then *in vitro* TBSV replicase activity assay was carried out using the microsome preparations and recombinant purified p33 and p92 replication proteins (Xu et al., 2012). Briefly, buffer A [50 mM Mg-acetate, 30 mM HEPES, 150 mM K-acetate, 13 mM sorbitol], 10 mM DTT, 0.1 mg/ml creatin kinase, 1 mM ATP, CTP and GTP, 0.025 mM UTP, 15 mM creatin phosphate, RNase inhibitor, DI-72 (+)repRNA, MBP-tagged, purified TBSV proteins p33 and p92, supernatant fraction of the CFE (obtained by ultra-centrifugation) and ER membrane fraction. The assays were done at 25 °C for 1 h. Then, additional reaction mix containing buffer B (50 mM K-Acetate, 30 mM HEPES, 5 mM Mg-Acetate), actinomycin D, DTT, creatin kinase, keratin phosphate, ATP, CTP and GTP, RNase inhibitor and ^{32}P-labeled UTP isotope was added and the reactions were incubated at 25 °C for 3 h (Xu et al., 2012). The obtained ^{32}P-labeled RNA products were analyzed on denaturing, 8M urea PAGE (Xu et al., 2012).

Membrane-based yeast two-hybrid assay

The split-ubiquitin assay is based on the Dualmembrane kit3 (Dualsystems) and performed as previously described (Li et al., 2008). *S. cerevisiae SEC*39 gene was amplified by PCR from gDNA using the primers #5222 (CCAGAGATCTATGTTGGAAGAGCAACTA-TATTTGTT) and #5223 (CCAGGCTAGCTCAGAAATTAGTCACGTT-GTGGA). The resulting PCR product was digested with *Bgl*II and *Nhe*I and ligated into similar digested pPR-N-RE and pPR-C-RE plasmids (Li et al., 2008). Yeast strain NMY51 was co-transformed with pGAD-BT2-N-His33 (Li et al., 2008) and pPR-N/C-RE (NubG, as a negative control) or one of the pPR host gene constructs and plated onto Trp^-/Leu^- (TL^-) minimal medium plates. Transformed colonies were picked with a loop, resuspended in water, serially diluted (from 1:1 to 1:10,000) and spotted onto $Trp^-/Leu^-/His^-/Ade^-$ ($TLHA^-$) plates to test for p33: Sec39p interaction. Plasmid expressing Ssa1p protein (pPR-N-SSA1) was used as a positive control as previously described (Barajas and Nagy, 2010).

Co-purification of Sec39-6xHA with Flag tagged p33 of TBSV

*SEC*39 of *Saccharomyces cerevisiae* was C terminally tagged on the chromosome with 6xHA epitope tag, using primer pairs #5462-S2 (CATACATACACCTTTATATAGCTATACGCTGTATGTATTCA-ATCGATGAATTCGAGCTCG) and #5463-S3 (GGCGGCATTGCTAGG-GAGATTTTCCACAACGTGACTAA-TTTCCGTACGCTGCAGGTCGAC) and Euroscarf pYM16–hphNT1 plasmid as suggested (Janke et al., 2004). The incorporation of 6xHA tag was confirmed by Western blot analysis using anti-HA antibody. Then, the yeast strain (SEC39-6xHA) was transformed with pESC-CUP1-HisFlag-p33 or pESC-CUP1-Hisp33 plasmids (*HIS*3 selection). Cells were grown in SC H^- glucose at 23 °C for 24 h by gradually increasing culture volume up to 200 ml. At $OD_{600} \sim 1.0$, the expression of p33 was induced with the addition of $CuSO_4$ (50 μM final concentration) and cells were grown for an additional 6 h. Next, we cross-linked the proteins with formaldehyde (final concentration 1%) and then quenched the reaction with glycine (final concentration 125 mM) (Barajas et al., 2009b). Then, cells were broken with glass beads in FastPrep®-24 instrument (MP Biomedicals), the membrane was solubilized with nonionic detergent and p33 was purified via anti-FLAG M2-agarose affinity resin (Sigma) as described previously (Barajas et al., 2009b; Sasvari et al., 2011). The bound proteins were eluted with 50 μl SDS-protein sample loading buffer without β-mercaptoethanol. After elution, β-mercaptoethanol was added and samples were boiled in water bath at 95 °C for 30 min to reverse cross-linking. The samples were analyzed by SDS-PAGE and Western blotting.

Confocal laser microscopy analysis

BY4741 and sec39-2^{ts} yeast strains were transformed with pESC-GAL1-CFP-p33/GAL1-DI72 (*his*3 selection) (Wang et al., 2009a) together with pGAD-ADH1-PHO86-YFP (*leu*2 selection) plasmid (Panavas et al., 2005a) that expresses YFP-tagged Pho86p ER marker protein. Cells were grown in SC LH^- glucose media at 23 °C overnight, then shifted to 32 °C for 3 h. Then, the culture media was changed to galactose to launch p33 expression for 3 h. Then, the culture media was changed back to SC LH^- glucose to stop expression of p33 and cells were grown for an additional 3 h. The live samples were subjected to confocal laser microscopy analysis (Jonczyk et al., 2007; Pathak et al., 2008).

THC-SEC39 and R1158 strains were transformed with pESC-CUP1-GFP-p33 (*his*3 selection) and pGAD-ADH1-Pho86-RFP (*leu*2 selection). Yeast cells were grown in SC LH^- glucose media containing 100 μM BCS in the presence or absence of doxycycline for 10–12 h at 28 °C. Then, BCS was removed and 50 μM $CuSO_4$ was added to induce expression of p33 for 3 h. Then, p33 expression was blocked by adding BCS to the cultures, and after additional 3 h of incubation, samples were subjected to confocal laser microscopy analysis. In the case of doxycycline (+) samples, doxycycline was present in the media throughout the growth period.

THC-SEC39 and R1158 strains bearing chromosomally GFP-tagged *PEX*3 (Perry et al., 2009) were transformed with pGAD-CUP1-RFP-p33 (*leu*2 selection). Growing conditions and confocal laser microscopy analysis were the same as above.

Virus induced gene silencing of ACC64519.1 Sec39-type gene in N. benthamiana

Human and *Arabidopsis* proteins with Sec39 domain were identified (A2RRP1, F4KH34, UniProtKB/Swiss-Prot identification numbers and NBAS, At5g24350.1 gene names, respectively.) The two orthologs, *Arabidopsis* F4KH34 and human A2RRP1 are 23% identical and the *SEC*39 domain follows WD40 domains in both cases. Human Sec39-type protein was shown to physically interact with ZW10, the human homologue of *Saccharomyces* Dsl1p, and RINT1, the human homologue of *Sc*Tip20p (Hutchins et al., 2010), which proteins are in the same complex as Sec39p in yeast. The sequence of *N. tabacum* SEC39 type gene (GenBank: ACC64519.1) was derived via BLASTP search of the *Arabidopsis thaliana* Sec39-type protein coded by At5g24350.1.

Virus induced gene silencing (VIGS) in *N. benthamiana* was performed as described in (Jaag and Nagy, 2009). To create the VIGS vector (pTRV2-SEC39A2*Nt*), a 301-bp cDNA fragment of ACC64519.1 *SEC*39-type gene was RT-PCR amplified from a total RNA extract using the primer pairs: #5567F (CGCGGATCCTCC-AACAAAAGTAAGGAGATATGC) and #5568R (CGCCTCGAGCCGT-CAGGCCAACTATTGAT) harboring restriction sites *BamH*I and *Xho*I

respectively and ligated to the pTRV2 plasmid digested with BamHI and *Xho*I.

To confirm the silencing of Sec39-type gene in *N. benthamiana* we performed RT-PCR amplification with primer pairs: #5404F and #5405R on total RNA extract of pTRV2-SEC39A*Nt* and $pTRV2_{empty}$ agroinfiltrated plants. RT-PCR analysis of *SEC*39 mRNA level in the silenced and control plants was based on 31 PCR cycles. Tubulin mRNA control was performed with RT-PCR using primer pairs: #2859 (TAATACGACTCACTATAGGAACCAAATCATT-CATGGTTGCTCTC) and #2860 (TAGTGTATGTGATATCCCACCAA). RT-PCR analysis of tubulin mRNA level in the silenced and control plants was based on 28 PCR cycles. VIGS-treated plants were sap inoculated with TBSV on the 9th day after silencing. Total RNA was extracted 2 days post inoculation and Northern blot analysis was carried out with ^{32}P-labeled RNA probe specific to the 3′ end of the viral genomic RNA.

Acknowledgments

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